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TEMPORAL RELATIONSHIP OF THE INDUCTION OF
TOLERANCE AND PHYSICAL DEPENDENCE AFTER
CONTINUOUS INTOXICATION WITH MAXIMUM
TOLERABLE DOSES OF ETHANOL IN RATS

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
BETHESDA, MARYLAND

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) In an effort to study chronic toxicity of the nervous system by chemicals, explora- tion of an animal model of ethanol dependence was examined. Previous work had demonstrated that physical dependence on ethanol could be induced in only a few days. The purpose of this study was to establish the existence of tolerance and to determine the rate of development of tolerance and physical dependence. These results demon- strate that when rats are treated with the maximum tolerable doses of ethanol, both		

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tolerance and physical dependence can be induced in only a few days. Animals were treated by intragastric intubation of a 20 percent ethanol solution in doses of 9-15 g/kg in three to five fractions for 1-7 days. Ethanol doses were determined individually for each animal utilizing intoxication-dose relationships. Tolerance was assessed by correlating the signs of intoxication with the descending blood ethanol concentration during the withdrawal period. The severity of intoxication was measured by signs which were related directly to the blood ethanol concentrations: coma, loss of righting reflex, ataxia-3, ataxia-2, ataxia-1, sedation and neutrality. As blood ethanol concentrations approached 100 mg/dl the ethanol dependence phase emerged which was characterized by the onset of signs and responses of progressive severity: hyperactivity, tremors, spastic rigidity and spontaneous convulsive seizures. As the duration of ethanol treatment increased beyond a single dose, the blood ethanol concentration for a given sign of intoxication also increased. A significant degree of tolerance was demonstrated for all signs of intoxication after 4 days of treatment, but did not reach maximum level even after 7 days. As the duration of ethanol treatment increased, the severity of the withdrawal reactions intensified progressively to a maximum intensity after 4 days of treatment when as many as 72 percent of animals exhibited severe withdrawal signs and reactions including convulsive seizures. The different time courses of development of tolerance and physical dependence might suggest that the two phenomena are mediated through different mechanisms. The results provide further information on the development and exploration of models for chronic insults to the brain, such as long-term exposure to toxic chemicals and ionizing and nonionizing radiation.

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TABLE OF CONTENTS

	Page
Introduction	3
Methods	4
Results	7
Tolerance	7
Physical dependence	7
Discussion	7
References	13

LIST OF FIGURES

Figure 1. Time course of acquisition of tolerance to ethanol following sustained intoxication with high doses of ethanol	8
Figure 2. Time course of acquisition of physical dependence on ethanol following sustained intoxication with high doses of ethanol	9

LIST OF TABLES

Table 1. Dosages of Ethanol in g/kg Given to Rats Throughout Various Periods of Time	4
Table 2. Percentage of Animals in Five Separate Categories of Withdrawal Reactions When Ethanol was Removed After Different Days of Treatment	9

INTRODUCTION

The development of tolerance and physical dependence on ethanol in humans and laboratory animals is well documented.^{4-6,11,13,15,16,19,24,25,35,37,41-43} Tolerance exists when the effect of a given dose of ethanol is reduced after chronic treatment with ethanol. Physical dependence, on the other hand, is evident upon withdrawal from chronic treatment by the appearance of a series of neurological signs and symptoms which include hyperirritability, tremors, hallucinations, convulsions and delirium tremens.^{7,19,21,29,35,38,43}

Recently, a model of physical dependence on ethanol in the rat was established²⁷⁻²⁹ displaying many of the signs and responses of the ethanol withdrawal syndrome observed in man.^{7,19,29,35,43} In order to induce physical dependence, a minimum concentration of ethanol in the blood had to be maintained 24 hours a day for several days. Also, changes in the excitability of the central nervous system after withdrawal represented a continuum of different signs and responses as the animal went through the process of detoxication and the withdrawal syndrome.^{28,29}

In the present study, this animal model of ethanol dependence was further characterized by exploring the relative rates of the development of tolerance and physical dependence. Measurements of tolerance have involved a variety of physiological and locomotor functions requiring experimental designs which make large scale behavioral evaluations in a short period of time impractical.⁹ As Kalant et al.²¹ have pointed out, most studies of tolerance have been designed using a single behavioral test or physiological measure. Frequently used indices for measuring tolerance to depressant drugs are the time of induction and the duration of the loss of righting reflex in animals. However, the restriction of the measurement of tolerance to the loss of righting reflex imposes a number of limitations for the experiments. This procedure usually requires large doses of drugs which frequently are on the borderline with coma and death. Secondly, loss of righting reflex is only one of an entire spectrum of neuromuscular signs which are impaired following the administration of

ethanol. Therefore, in the studies described in this paper we evaluated the onset of tolerance in treated animals by using a spectrum of intoxication signs ranging from neutrality to coma.^{4,6,7,18,29,38,41}

A similar approach can be taken when studying the rate of development of physical dependence. Previous reports have generally employed only one sign of withdrawal. These have included determinations of the susceptibility to seizure^{8,15,17,32,39} and the utilization of behavioral endpoints^{1,10} and cortical electrical activity.¹⁸ Freund⁶ and Ellis and Pick⁴ examined a number of withdrawal signs but did not study the time course of physical dependence. Therefore, in this study a broad range of signs and responses have been used to describe the various changes observed during the development of the ethanol withdrawal syndrome.

METHODS

Male Sprague-Dawley rats (200-350 g) were treated with a 20 percent (w/v) aqueous solution of absolute ethanol by means of intragastric intubation. Ethanol was administered in doses of 9-15 g/kg per day in three to five fractions for 1-7 days. In Table 1 are shown the means and standard deviations of the daily doses of ethanol given to rats throughout various periods of treatment. Each experiment was initiated with the administration of a priming ethanol dose

Table 1. Dosages of Ethanol in g/kg Given to Rats Throughout Various Periods of Time

Day of Treatment	Duration of Treatment (Days)					
	1	2	3	4	5	7
Daily mean ± S.D.	11.5 ± 2.8	10.2 ± 1.9	9.7 ± 2.4	9.9 ± 2.4	9.5 ± 2.2	9.3 ± 2.9
Last dose	4.1 ± 1.3	4.4 ± 1.8	4.1 ± 1.4	4.0 ± 1.5	4.0 ± 1.5	3.1 ± 1.1
Number of rats	37	35	53	93	67	27

of 5 g/kg. Subsequent doses were determined individually for each animal utilizing the previously described correlation between the blood ethanol concentrations and the severity of intoxication.^{28,29} Accordingly, rats rated neutral received 4 g/kg; ataxia-1, 3 g/kg; ataxia-2, 2 g/kg; and ataxia-3, 1 g/kg. Animals which displayed loss of righting reflex or were comatose received no ethanol. Since after a few days of treatment the animals developed considerable tolerance, the individual doses of ethanol were somewhat increased.²⁹ However, the total daily amount of alcohol given to the animals remained unaffected (Table 1). On the day of ethanol withdrawal, the animals were allowed to detoxify and the degree of intoxication assessed at hourly intervals. Blood samples for ethanol determinations were drawn immediately after each behavioral evaluation. The degree of intoxication was assessed in a subjective fashion using the signs and responses described by Majchrowicz^{28,29} and briefly stated below:

- | | |
|-------------------------|---|
| Neutrality | - no overt signs of intoxication |
| Sedation | - reduced muscle tone, dulled and relaxed appearance, slow locomotor activity, no overt gait impairment or motor incoordination |
| Ataxia-1 | - lowest degree of gait impairment and motor incoordination, abdomen and pelvis still markedly elevated |
| Ataxia-2 | - responses falling between ataxia-1 and ataxia-3, accentuated staggering gait |
| Ataxia-3 | - slowed righting reflex, heavily impaired motor coordination, absence of pelvic and abdominal elevation |
| Loss of righting reflex | - inability of animal to right himself, other reflexes still present, little or no spontaneous motor activity |
| Coma | - no signs of movement and completely unresponsive, absence of eyeblink reflex. |

The severity of the withdrawal syndrome was assessed on the basis of the appearance of at least three behavioral signs.²⁷⁻²⁹ The signs used in this study are listed as follows:

General

hyperactivity - increased locomotor activity, accentuated startle response, agitation

Tremors - can involve the tail, caudal region, trunk, head, or the whole body

Rigidity - expressed as curling of the tail around a stiff rod or increased muscle tone in the body characterized by the maintenance of rigid posture

Convulsive seizures - spontaneous and induced tonic-clonic.

The occurrence of convulsive seizures automatically classified an animal as severe, regardless of the intensity of other signs of the withdrawal syndrome. When all of the other three signs were present, the intensity of the withdrawal syndrome was also rated severe. When any three of the symptoms were just observable, the intensity was rated mild. The appearance of signs intermediate between severe and mild resulted in a moderate rating.

In acute studies single doses of ethanol were administered at a rate of 5 g/kg. The severity of intoxication was determined at hourly intervals in the manner just described until the blood ethanol concentration declined to below 100 mg/dl. Blood samples were taken immediately after each behavioral evaluation. Acute tolerance has been observed after administration of single doses of ethanol^{19,23,33,36} where similar signs of intoxication were observed at lower blood ethanol concentration on the ascending limb than on the descending limb of the blood ethanol curve. Therefore, in order to standardize the experimental procedure, all evaluations of the degree of intoxication were made while blood ethanol declined and during the detoxication phase of the withdrawal period, while the blood ethanol concentrations were decreasing.

Blood was taken from the tail vein³⁰ and analyzed for ethanol by an automated adaptation²⁶ of the gas chromatographic method of Roach and Creaven.⁴⁰

RESULTS

Tolerance. Administration of heavy doses of ethanol for prolonged periods of time resulted in the development of tolerance to the effects of ethanol (Figure 1). The induction of tolerance to ethanol increased gradually as the length of the treatment was prolonged as shown using linear regression analysis ($P < 0.05$). The tolerance can be seen by the increase in blood ethanol concentrations as the duration of treatment increased. Statistically significant differences using Student's "t" test ($P < 0.05$) were found for sedation, ataxia-1 and ataxia-3 after as little as 1 day of treatment. Tolerance was demonstrated for all signs of intoxication after 4 days.

Physical dependence. The severity of the withdrawal syndrome intensified progressively as the period of treatment with ethanol increased (Table 2). After a single day of treatment a majority of the animals (55 percent) displayed at least mild withdrawal signs. Sixteen rats (42 percent) showed mild signs, while only one developed convulsions. On the basis of successive χ^2 analyses using 2×4 contingency tables, there was a significant trend toward increased withdrawal scores with each additional day of treatment. After 4 days of treatment maximum intensity of the withdrawal syndrome was observed with as many as 72 percent of the rats exhibiting severe withdrawal reactions including convulsions (Table 2, Figure 2). Continuation of treatment for longer periods of time did not result in consistent accentuation of the withdrawal reactions.

DISCUSSION

The results of this study demonstrate that when rats are treated with the maximum tolerable doses of ethanol, tolerance and physical dependence can be induced in only a few days. In fact it is possible to observe significant signs of both phenomena after as little as 1 day of treatment. This is in contrast to the generally held view that physical dependence in alcoholic persons can be induced only after prolonged consumption of large doses of ethanol.^{19,20,43} However, with the use of animal models physical dependence can be induced in a

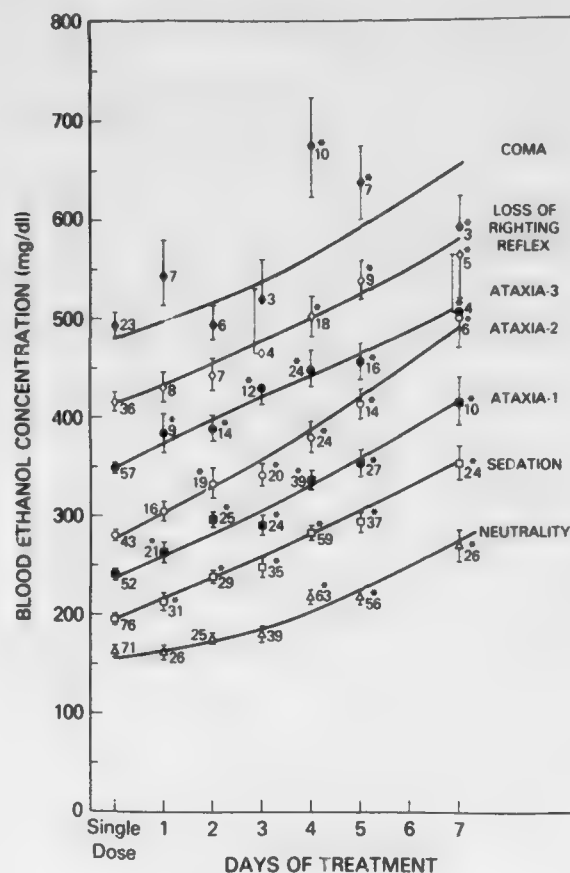


Figure 1. Time course of acquisition of tolerance to ethanol following sustained intoxication with high doses of ethanol. All observations were made during the ethanol detoxication phase of the withdrawal period which corresponds to the descending limb of the blood ethanol curve. Each point represents the mean blood ethanol concentration \pm S. E. at the onset of a given sign of intoxication during the ethanol detoxication phase of the withdrawal period or after a single intubation with ethanol (5 g/kg). The numbers beside the blood ethanol points indicate the number of observations used for the assessment of the signs of intoxication. The asterisk (*) denotes statistical significance from control using Student's "t" test ($P < 0.05$). Linear regression analysis of the data for sedation, ataxia-1, ataxia-2, ataxia-3 and loss of righting reflex showed a significant upward trend ($P < 0.05$). This analysis was not performed for the data for neutrality and coma either because of insufficient data or lack of linearity.

Table 2. Percentage of Animals in Five Separate Categories of Withdrawal Reactions When Ethanol was Removed After Different Days of Treatment

Days of Treatment	Total Number of Rats	Percentage of Animals in each Designated Withdrawal Category				
		0 No Withdrawal Signs	1 Mild	2 Moderate	3 Severe; Convulsions only	4 Severe; Excluding Convulsions
1	38	45	42	11	3	0
2	36	22	22	22	10	17
3	47	8	17	19	34	21
4	119	■	11	17	29	43
5	89		14	15	29	34
6	29	■	10	35	24	23
7*	57	2	39	28	17	15

*The total percentage ≠ 100 due to rounding of individual values

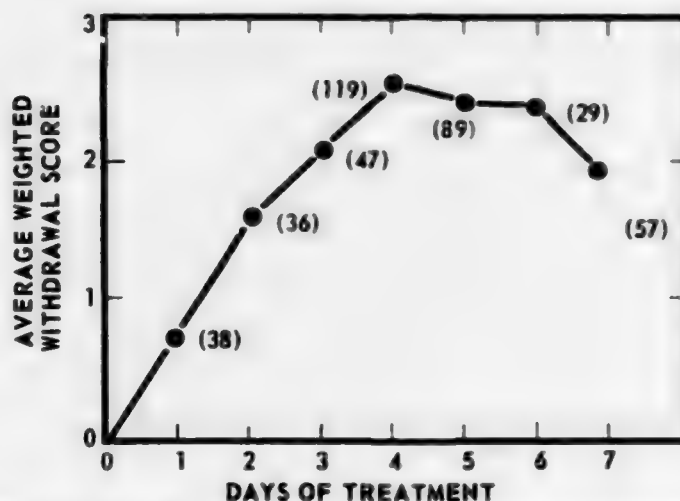


Figure 2. Time course of acquisition of physical dependence on ethanol following sustained intoxication with high doses of ethanol. The average weighted withdrawal score was obtained by multiplying the number of animals given a certain withdrawal score by that score and dividing by the total number of animals for each day of treatment (see Table 2). The numbers in parentheses denote the total number of animals used. χ^2 analyses between each successive day of treatment demonstrated a significant upward trend in the withdrawal scores up to 4 days ($P < 0.05$).

matter of days.³⁴ In light of our work and that of Freund⁶ and Goldstein¹⁴ it appears as suggested earlier by Seevers and Deneau⁴¹ that the length of time necessary for physical dependence to be expressed as a withdrawal syndrome depends on the total amount of ethanol consumed per day and the continuous distribution of the doses over a 24-hour period. Freund⁶ was only able to induce ethanol dependence using a liquid diet which attracted the animals to drink continuously. If a 10 percent ethanol solution was used where consumption was more variable and less stable, no withdrawal syndrome was observed. Goldstein¹⁴ has shown, using the vapor chamber technique for administering ethanol, that interrupting the continuous administration of ethanol for short periods resulted in a decay of physical dependence with complete loss after 24 hours. In alcoholic men under laboratory conditions a withdrawal syndrome has been demonstrated after a week of continuous drinking.^{31,35} One reason why it might take longer for alcoholic people in society to induce a state of physical dependence is their inability to sustain severe degrees of intoxication for prolonged periods of time without rendering themselves unconscious. In our experiments, the rats were severely intoxicated and were still given ethanol doses at a time when alcoholic subjects might not be able to drink. The conclusion to be drawn is that in order to attain a certain level of physical dependence, a given blood concentration of ethanol must be maintained as nearly to 24 hours a day as possible and for a requisite number of days.⁴¹ If the blood ethanol concentration is reduced or the even distribution of dose is interrupted, the time required to induce the same degree of physical dependence will be correspondingly increased. If this pattern of ethanol administration is distorted beyond a critical point, little or no physical dependence will be observed. Conversely, continuously sustained intoxication with high doses of ethanol can induce dependence in a very short time.

Considerable tolerance was observed after as little as 1 day of treatment with ethanol using as an end point seven different levels of intoxication correlated with the corresponding blood ethanol concentration. Over a period of

7 days, the blood ethanol concentration observed for each of these behavioral categories increased from 56 to 85 percent. This compares favorably with studies reported previously.^{2,22,44} LeBlanc et al.²² also observed equivalent tolerance over the same time period, but used much smaller doses. Since the highest tolerable dose of ethanol was used throughout the induction period, the degree of tolerance observed for each day of treatment is presumed to be maximal. Considering that the blood ethanol concentration increases less than two-fold over the period studied for any particular sign of intoxication, the tolerance observed is considered of low grade. This is in contrast to the development of tolerance to narcotic analgesics where a dose of morphine given to a tolerant rat is about four to seven times larger than to a nontolerant one.⁴⁵

One question of interest is whether the development of tolerance and physical dependence is mediated by the same mechanism, i. e., are tolerance and physical dependence different expressions of the same insult to the brain by chronic ethanol treatment? Theories of Goldstein and Goldstein¹² and Collier³ have involved a unitary mechanism to explain both phenomena. Briefly stated, when a drug depresses the nervous system for a sufficient length of time, adaptive mechanisms are engaged which raise the level of excitability to antagonize the depressant actions. This can be done to the point where the drug no longer has any pharmacological effect. If the drug treatment is abruptly discontinued and the depressant effect removed, the net effect is a higher level of excitability than found initially. Hence, a withdrawal syndrome develops.

Our data suggest that tolerance and physical dependence do not develop through a common mechanism. Maximal physical dependence by our criteria was observed after 3-4 days of treatment (Figure 2), while maximal tolerance was not obtained even after 7 days (Figure 1). The durations of the two reactions are different. Physical dependence decays within 24-48 hours using the symptoms of the withdrawal syndrome as an end point.^{14,29} Tolerance, on the other hand, can be observed for 3 weeks after withdrawal.²⁴ Finally, treating

animals chronically with one dose of ethanol per day can induce maximum tolerance without evidence of the development of physical dependence.^{24,37}

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